DISSERTATION ABSTRACT

学位論文題名: **TIME PACING IN CHONDROGENESIS MEDIATED BY THE CLOCK GENE *PERIOD 1***

(和訳): 時計遺伝子ピリオド 1 による軟骨細胞分化の周期的制御

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Summary

During postnatal development, most bones of vertebrates grow in length through a process of cartilage replacement with bone within embryonic cartilaginous remnants, termed endochondral ossification. Various circulating hormones and cytokines coordinate to drive chondrogenesis through activity of mixture of transcription factors such as Sry-type high mobility group box proteins (Sox5, Sox6, and Sox9) and runt-related transcription factor protein (Runx2). Here, we have shown upregulation by parathyroid hormone (PTH) of the clock gene Period 1 (*Per1*) through the PKA/cAMP signaling pathway in pre-chondrocytic ATDC5 cells. Hence, our investigation was prone to the role of *Per1* in the suppression of chondrogenic differentiation by PTH. In ATDC5 cells exposed to 10 nM PTH, a drastic but transient increase in *Per1* expression was obtained only 1 h after addition together with a prolonged decrease in *Sox6* levels. However, no significant changes were induced in *Sox5* and *Runx2* levels in cells exposed to PTH. In stable *Per1* transfectants, a significant decrease in *Sox6* mRNA levels was seen, with no apparent alterations in *Sox5* and *Sox9* mRNA levels, in addition to the inhibition of gene transactivation by Sox9 allies. In reversal, knocking down *Per1* by siRNA apparently increased the *Sox6* and type II collagen transcription in ATDC5 cells of 24 to 60 h in culture. These results suggested that the clock gene *Per1* might play a suppressive role in chondrocytic differentiation, which was stimulated by PTH through a mechanism relevant to negative regulation of transactivation of the *Sox6* gene during chondrogenesis. Additionally, the information obtained in this study would be helpful in understanding clock systems in various cartilages *in vivo.*

CHAPTER I. GENERAL INTRODUCTION

1.1. Circadian rhythms

A circadian rhythm is an endogenous rhythm whose every cycle length is approximately 24 hours (h) and has existed in many kinds of living things, from unicellular prokaryotic to multicellular eukaryotic animals (Herzog, 2007).

1.1.1 Circadian oscillators

Circadian oscillators (pacemakers) generate a rhythm which repeats with a frequency of about 24 hours. Therefore, a circadian rhythm should possess several fundamental features: (*i*) they are endogenously generated oscillations with cycles of about 24 h, (*ii*) they exhibit homeostasis of period, (*iii*) with temperature compensation, and (*iv*) they are synchronized by periodic environmental signals, or entrainable to external cues (Rosbash, 1995).

1.1.2 The mammalian circadian oscillator

1.1.2.1. The central oscillator – The SCN

The suprachiasmatic nucleus, a bilaterally paired nucleus in the mammalian hypothalamus and ablation of the SCN made circadian rhythms of many physiological and behavioral activities to be vanished (Pittendrigh, 1972).

1.1.2.2. SCN anatomical properties

Each nucleus can be subdivided into two part: the ventrolateral or core (vlSCN) and the dorsomedial or shell (dmSCN) (Moore et al., 2002).

1.1.3 Peripheral oscillators – a hierarchical organization

In mammals, the SCN is believed to orchestrate peripheral clocks to exert its synchronization at cellular level in a particular tissue. Peripheral tissues could become uncoupled to the SCN rhythmicity by resetting time under the influence of external cues, thus, gene expression become shifted (Damiola et al., 2000; Schibler et al., 2003).

1.1.4 Biological clock under molecular scope

1.1.4.1. The core loop – heart of the circadian oscillator

The core-loop components of mammalian clockwork has been revealed as follows: circadian locomotor output cycles kaput or *Clock*, brain and muscle-ARNT like protein 1 or *Bmal1* (MOP3), neuronal-PAS domain protein 2 (*NPAS2*), period homologs *Per1, Per2, Per3*, Cryptochrome homologs *Cry1* and *Cry2*.

1.1.4.2. Circadian gene regulation – the interlocked loops

To date, four *cis*-reglatory elements that are known to participate in the circadian regulation: E-box, DBP/E4BP4 binding elements (D-box), REV-ERBα/RORα binding elements (RORe) and cyclic AMP responsive elements (CRE) (King et al., 1997; Ginty et al., 1993; Ripperger and Schibler, 2006; Ueda et al., 2005) (Fig.1.1).

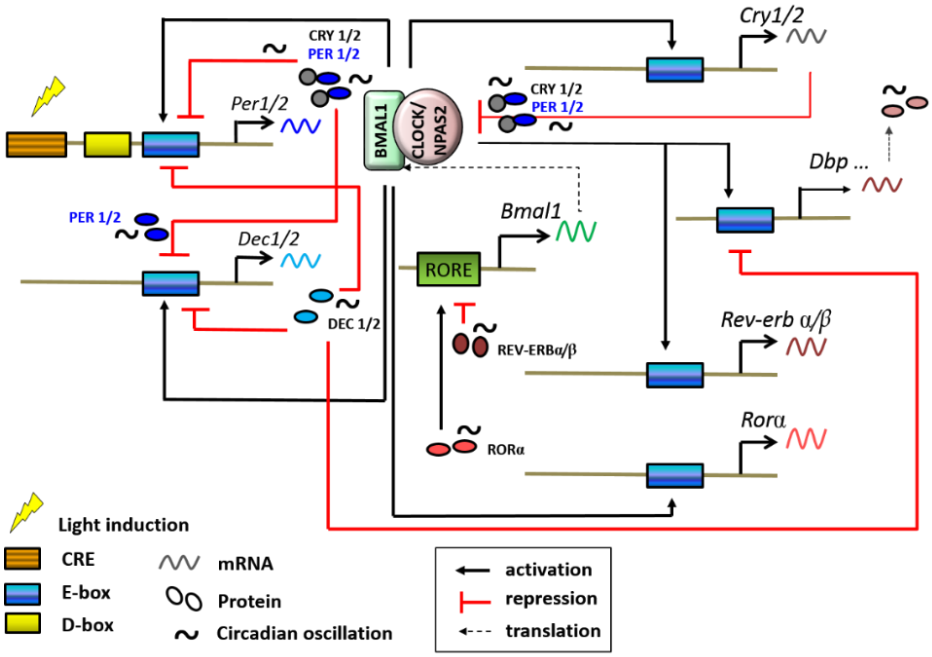

Figure 1.1. The clockwork regulation. The CLOCK(NPAS2)/BMAL1 heterodimer is at the heart of the cycle.

1.2. Cartilage and bone growth

1.2.1. The Bone

The basic multicellular unit of bone comprises the osteocytes, osteoclasts and osteoblasts. Bone formation begins when mesenchymal cells form condensations or clusters of cells. These cells either differentiate directly into bone forming osteoblasts (*intramembranous ossification* of flat bones, e.g. skull, scapula, ileum) or dominantly into chondrocytes that lay down a cartilage mold and is subsequently replaced by ossified bone (*endochondral ossification* of long bones, e.g. tibia, humerus, femur) (Karsenty, 2003).

1.2.2. The cartilage

Cartilage is a connective tissue of predominantly mesodermal origin that provides mechanical support and structural form to many areas of the musculoskeletal system. During embryonic development, mesenchymal stem cells (MSCs) form two types of cartilage: *permanent* and *transient* cartilage (Kronenberg, 2003).

1.2.3. Chondrogenesis regulation

1.2.3.1. Chondrocytes – developmental units of cartilage

Within the normal growth plate, chondrocytes are organized into layers and very responsive to various chemical mediators including hormones, growth factors and cytokines, as well as mechanical and possibly electrical stimuli.

1.2.3.2. Chondrogenesis

Chondrogenesis initiates with a hallmark in mesenchymal condensation facilitated by multi signals: different transcription factors activated via the Wnt, BMP, Sonic Hedgehog (Shh), and Indian Hedgehog (Ihh) (Macsai et al., 2008) and hormones (Locker et al., 2004). Thus, *the rate of chondrocyte differentiation* has to be carefully regulated so that, ultimately, *the proper shape and length* of the bone are both achieved and maintained.

1.2.4. Chondrogenesis – *in vitro* studies

ATDC5 cell line, isolated from a differentiating culture of AT805 teratocarcinoma cells, had shown a lot identical properties to chondroblasts *in vivo*, and was able to produce cartilage nodule-like aggregates to ultimately chondrogenic differentiation at a very high percentage compared to others cell lines, C3H10T1/2 and RJC3.1 (Atsumi et al., 1990; Shukunami et al., 1996).

1.3. Clockwork integration into bone and cartilage development

Circadian clock genes were found to harbor in bone and cartilage and modulate them (Tonna et al., 1987; Iimura et al., 2012; Vitaterna et al., 1999; Honda et al., 2013).

1.4. Aims of the dissertation

(i) To establish an *in vitro* chondrogenesis model using ATDC5 cell line for observing possible changes utilizing gain/loss-in-function of a particular clock gene, *Per1*, and

(ii) Thereby, to unravel the mysteriously underlying mechanism of clock gene *Per1* alteration towards chondrogenesis.

1.5. Abbreviations

1.6. Cell culture and Reagents: All in use were at a special analysis grade available commercially.

1.7. Data statistical analysis

Each result was expressed as mean ± SE. and statistical significance was determined by two-tailed and unpaired Student's t-test/ one-way analysis of Variance (ANOVA) with Bonferroni/Dunett post hoc test.

CHAPTER 2. PTH INDUCES THE INCREASE IN *PER1* EXPRESSION

2.1. Introduction

2.1.1. Parathyroid hormone pharmacological properties

PTH maintains calcium homeostasis which is crucial for a vast of essential cellular processes. Common outcome for PTH/PTH receptor-1 transduction is involved in G-protein coupling-dependent manner (Sakamoto et al., 2005).

2.1.2. PTH effects on bone

PTH is a key regulator in bone anabolic (Parfitt, 1976) and bone remodeling (Compston, 2007).

2.1.3. SOX family and chondrogenesis

Members evolved from the mammalian Sry encodes a nuclear factor-like protein harboring a DNA-binding domain known as the HMG box are namely Sox (SRY-related HMG-box) genes. *Sox9* plays a key role in successive of chondrogenesis (Akiyama et al., 2002; Bi et al., 2001). *Sox9* and two other Sox family group D, *Sox5*/L-*Sox5* (L-long form) and *Sox6* genes are synergetic allies in chondrogenesis during early stages as well as co-express in all embryonic cartilaginous primordial (Smits et al., 2001; Lefebvre et al., 1998) (Fig.2.1).

2.1.4. Runx family

Runx2 is essential for osteoblast differentiation and required for embryonic bone formation as well. Interestingly, *Runx2* is essential for differentiation of mesenchymal cells into osteoblasts, a combined action of *Runx2* and *Runx3* is essential for chondrocyte maturation, and endochondral ossification as well (Komori et al., 1997; Goldring et al., 2006) (Fig.2.1).

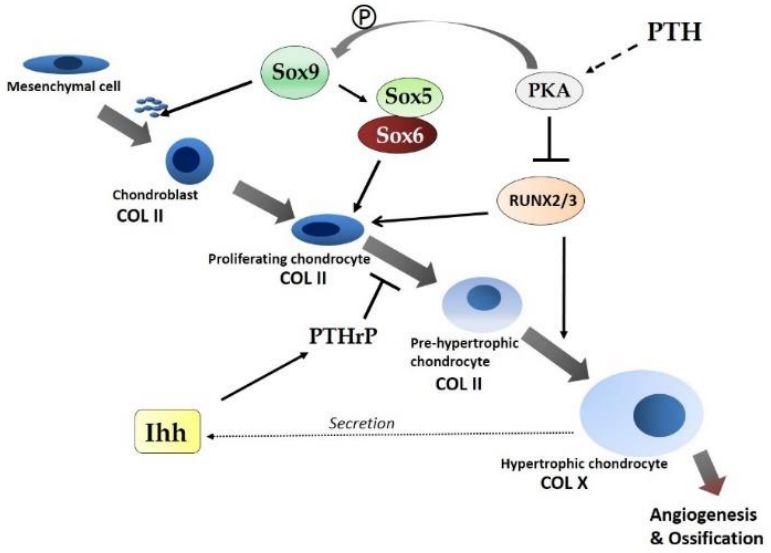

Figure 2.1. Chondrocyte differentiation. COL II: type II collagen; COL X: Type X collagen; Ihh: Indian Hegdhog; RUNX2/3: Runt-related protein 2/3; PKA: phosphate kinase A; PTH: parathyroid hormone.

2.2. Methods

2.2.1. ATDC5 cell culture

ATDC5 cells were plated at a density of 1×10^4 cells/cm_2 in DMEM/F12 medium containing 5% fetal bovine serum (FBS). For induction of differentiation, culture media were replaced with a medium formula containing 10 µg/ml transferrin, 3 x 10^{-8} M sodium selenite and 10 µg/ml bovine insulin (ITS).

2.2.2. PTH induction

Rat PTH (1-34) was added into the culture medium at the concentration of 10 nM for different periods.

2.2.3. Quantitative Real-time PCR (qPCR)

Table 2.1. qPCR primers

Gene	Primers	
	Forward	Reverse
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Per1	CAGGCTAACCAGGAATATTACCAGC	CACAGCCACAGAGAAGGTGTCCTGG
Runx2	CCGCACGACAACCGCACCAT	CGCTCCGGCCCACAAATCTC
Sox5	GATGGGGATCTGTGCTTGTT	CTCGCTGGAAAGCTATGACC
Sox6	GGATTGGGGAGTACAAGCAA	CATCTGAGGTGATGGTGTGG
Sox9	CGACTACGCTGACCATCAGA	AGACTGGTTGTTCCCAGTGC
Col II	TGAAGACCCAGACTGCCTCAA	AGCCGCGAAGTTCTTTTCTCC

Appropriate expression of mRNA was quantified by real time-based RT-PCR using a MiniOpticonTM (Bio-Rad, Hercules, CA, USA) with an iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The relative amount of transcript was normalized by glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression in a real time base and calibrated by fold changed when compared to none-induced culture. Primers used are listed in table 2.1.

2.3. Results

Exposure to PTH led to a rapid but transient increase in *Per1* mRNA expression 1 h after supplemented, with concomitant decrease of *Sox6* expression after the exposure for 12 to 48 h in ATDC5 cells (Fig.2.2). Nonetheless,

PTH induced a biphasic increase in *Sox9* levels during the exposure for 1 to 48 h, without significantly affecting the mRNA levels of *Runx2* and *Sox5*.

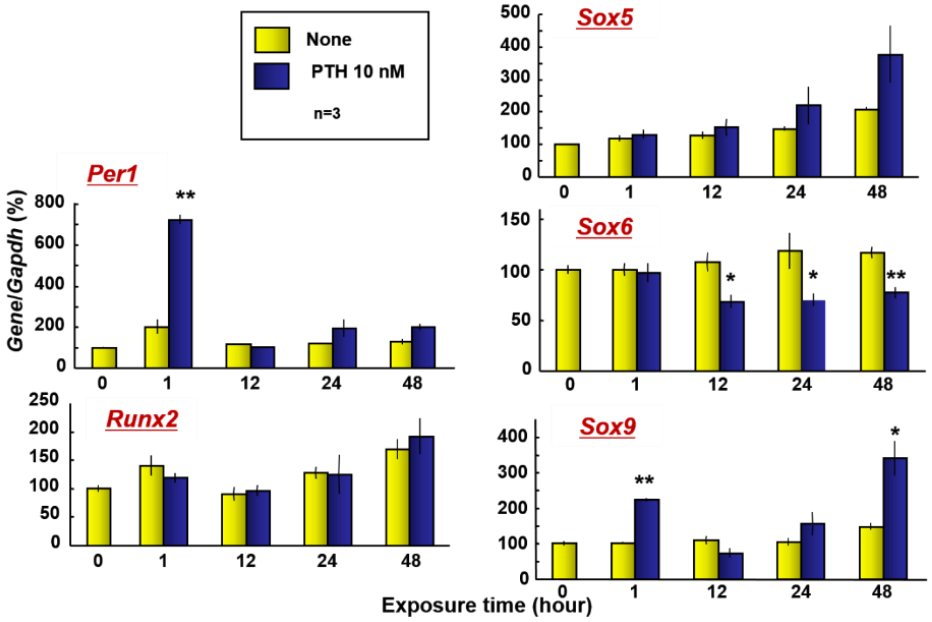

Figure 2.2. Expression profiles of different chondrogenic genes in ATDC5 cells exposed to PTH. ATDC5 cells cultured for 1 day were exposed to 10 nM PTH for 1 to 48 h, followed by isolation of total RNA and subsequent real time-based RT-PCR analysis. (*P<0.05, **P<0.01, significantly different from each control value obtained in cells cultured in the absence of PTH).

2.4. Conclusions

PTH has been demonstrated to have an effect on the rapid elevation of *Per1* mRNA in ATDC5 cell culture through the mechanism of G-protein coupling activation resulting in gene activation in the cAMP-PKA signaling responsive element binding transcription (Hinoi et al., 2006).

CHAPTER 3. THE UPREGULATION OF *PER1* EFFECTS ON CHONDROCYTIC DIFFERENTIATION

3.1. Clock gene *Per1* overexpression

Transcription regulation of *Per1* and *Per2* is believed via E-box and D-box embedded in promoter region. All five E-box plus three CRE-binding sites within *Per1* full-length promoter, synergistically affecting *Per1* transcription (Hida et al., 2000).

3.2. Promoter activation of Sox9 target genes during chondrogenesis

SOX9 protein can act on several chondrocytic genes through binding sites in their promoters. These binding sequences have been identified as enhancers of *Col II* (or *Col2a1*) (Lefebvre et al., 1996). A 48-bp element of intron 1 of mouse *Col II* gene that when exists in the form of 4 tandem copies conferred chondrocyte specific expression both in transgenic mice and in transient experiments of tissue culture cells (Zhou et al., 1998), which were cloned directly upstream of an 89-bp minimal sequence for *Col II* promoter (Murakami et al., 2000; de Crombrugghe et al., 2000).

3.3. Gene delivery in mammalian cells

For gene delivery in mammals, it has been reported the effective utilization of pcDNA 3.1 for gene overexpression in several culture systems. Gene transfection method adopted Lipofectamine 2000 is widely chosen for gene delivery in mammalian cells.

3.4. Methods

3.4.1. Establishment of stable *Per1* transfectants

ATDC5 cells were plated at a density of 2×10^4 cells/cm^2 in DMEM/F12 containing 5% FBS on culture dishes (Φ35 mm). After 24 h, cells were transfected with pcDNA3.1 vector containing the full-length coding region of *Per1* or with the empty vector (*E.V.*) using 2 µg of DNA and Lipofectamine 2000 and Plus reagent. After 24 h, and every 48 h thereafter for 2 weeks, culture media were replaced with DMEM/F12 containing 5% FBS and 500 µg/ml G418. Pools of 28 clones of ATDC5 cells resistant to G418 (ATDC5-*Per1*) were isolated for further studies (Uno et al., 2011).

3.4.2. Luciferase reporter assay

Reporter vectors (pGL3-basic-Luc) were co-transfected with a pSV40-*Renilla* luciferase (pSV40-RL-Luc) construct into ATDC5 cells using Lipofectamine 2000 and Plus reagent. 24 h after transfection, luciferase activity was determined according to the manufacturer's protocol (Uno et al., 2011).

3.4.3. Northern blot

Total RNA was extracted from cultured cells as described previously (Hinoi et al., 2003), then purified for Northern blot procedure as described elsewhere (Takarada et al., 2008).

3.4.4. qPCR and ATDC5 culture: qPCR was performed as described in **2.2.3** and ATDC5 cell culture was conducted as described in **2.2.1**.

3.5. Results

3.5.1. *Per1* was transfected stably into ATDC5 cells

ATDC5-*Per1* clone #28 stable transfectant (referred as ATDC5-*Per1* #28 hereafter) showed a significant increase in *Per1* mRNA level observed and cell survival.

3.5.2. Early chondrogenic genes in response to *Per1* overexpression

Real time based RT-PCR analysis again revealed high expression of *Per1* in the clone #28 cultured for 3 days, whilst unaltered *Runx2* level was seen (Fig.3.1A). Both ATDC5-EV and ATDC5-*Per1* #28 cells were then cultured in the presence of ITS for 7 days, followed by determination of the mRNA levels of *Sox9* allies with real time-based RT-PCR. Interestingly, stable overexpression of *Per1* was found to almost completely diminished *Sox6* expression without significantly affecting either *Sox5* or *Sox9* expression (Fig.3.1B).

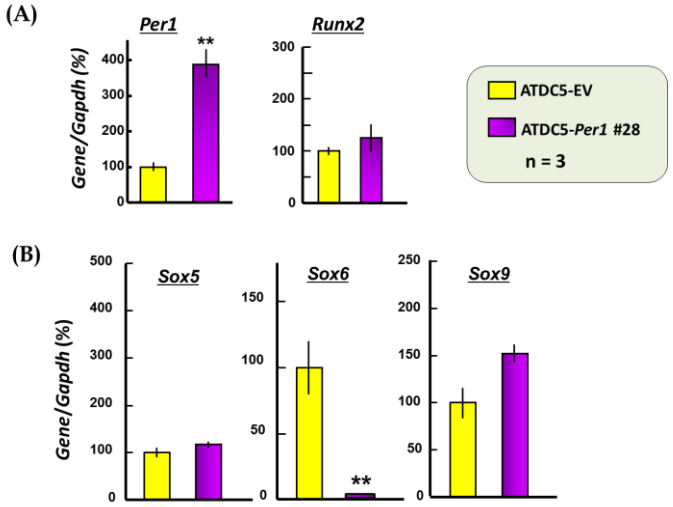

Figure 3.1. Stable overexpression of *Per1* in ATDC5 cells. (**P<0.01, significantly different from each control value obtained in cells with EV).

3.5.3. *Col II* transcription reduced in *Per1* stable transfectants

A 4x48-p89-Luc reporter plasmid linked to the minimal promoter of *Col II* (Fig.3.2A) was transiently transfected into ATDC5-*Per1* #28 (Fig.3.2B). Luciferase activity was significantly attenuated after 48 h transfection in ATDC5-*Per1* #28 cells compared to that in ATDC5-EV cells (Fig.3.2C). Northern blotting analysis confirmed a significant decrease in mRNA expression of the proliferating chondrocytic marker type II Collagen (*Col II*) in ATDC5-*Per1* #28 cells cultured for 7 days (Fig.3.2D).

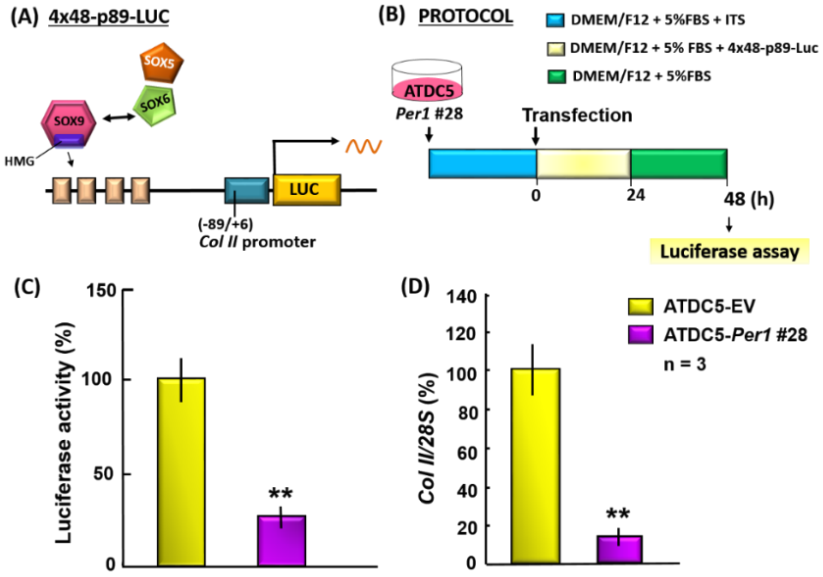

Figure 3.2. *Col II* transactivation (**P<0.01, significantly different from each control value obtained in cells with EV).

3.6. Conclusions

While *Per1* expression sharply increased, not only *Sox6* expressed a decreased mRNA expression level but lower that of *Col II* gene was obtained as well. The result implies that *Sox9* ability in transactivation on *Col II* gene was also reduced markedly.

CHAPTER 4. CHONDROGENIC DIFFERENTIATION IN COORDINATION WITH *PER1* REPRESSION

4.1. Gene silencing by short-interference RNA

4.1.1. siRNA processing

The siRNA processing undergoes some major steps resulting in specific mRNA cleavage.

4.1.2. Systematic nature of silencing

siRNAs have been involved in almost all possible nucleic acid regulatory pathways like target cleavage, transcriptional gene silencing and DNA elimination.

4.1.3. Gene Knock down by SiRNA delivery in vitro

siRNAs has great potential in the field of knock-down gene research in mammalian cells (McManus and Sharp, 2002).

4.2. Methods

4.2.1. Transfection ATDC5 cells with *Per1*-siRNA

ATDC5 cells were plated at 2 x 10^4 cells/cm^2 in DMEM/F12 in the presence of 5% FBS 24 h before transfection in Φ35 mm dishes. siRNA was introduced into cells at 30 nM by Lipofectamine 2000 for 24 h and subsequent replacement of the medium to DMEM/F12 containing 5% FBS. BLOCK-i™ Alexa Fluor Red Fluorescent Oligo and Silencer GAPDH siRNA (Human, Mouse, Rat) was used for the evaluation of siRNA transfection efficiency in ATDC5 cells.

4.2.2. ATDC5 cell culture: performed as described in **2.2.1**

4.2.3. qPCR: performed as described in **2.2.3**

4.2.4. Western blot

4.2.4.1. *Cell lysate preparation*: treated with reagent, followed by protein denaturing for 5 min at 100^{0}C.

4.2.4.2. *Polyacrylamide gel electrophoresis (SDS-PAGE)*: 10% polyacrylamide gel.

4.2.4.3. *Protein blotting*: Protein blotting using PVDF membrane, then transferring for 30 min hours at r.t. at transferred current (1.6 mA/cm2). Stain with 1x Ponceau S and rinse in ultra-pure H_2O (ddH2O).

4.2.4.4. *Blocking – Incubation with antibodies – Detection*: Incubating the membrane with dilute primary antibody O/N at 4^{0}C. For HRP conjugated primary antibody, incubated for 1 hour at r.t. Ultimately, detecting protein signal using ECL kit.

4.2.5. Luciferase assay: performed as described in **3.4.2.**

4.3. Results

4.3.1. *Per1* was down regulated in siRNA transfected cells

ATDC5 cells were transfected with small interfering RNA (siRNA) for the knockdown of *Per1* expression (Fig. 4.1A). Approximately 90% of cells were successfully transfected with siRNA conjugated with Alexa594 in ATDC5 cells transfected under the present experimental conditions (Figure 4.1B). The *Per1* mRNA levels were significantly decreased in ATDC5 cells transfected with *Per1* siRNA when determined at 24, 36 and 48 h, but not at 60 h after the transfection (Figure 4.1C). In cells transfected with *Per1* siRNA, a marked reduction of PER1 protein was found 60 h after transfection (Fig. 4.1D).

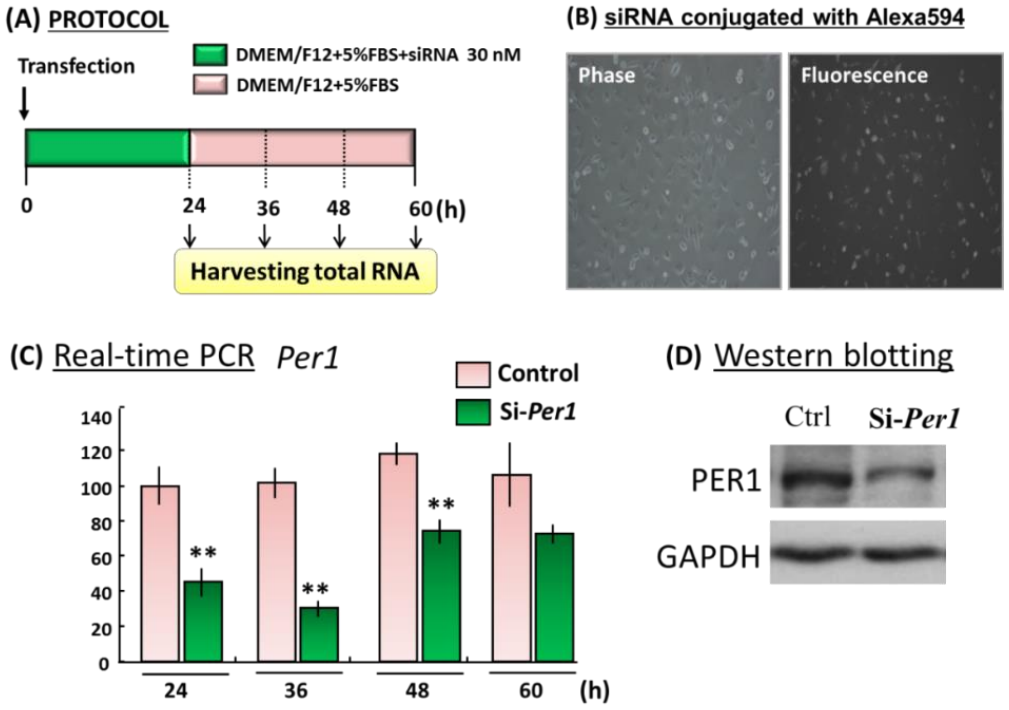

Figure 4.1. Knockdown of *Per1* in ATDC5 cells by siRNA (*P<0.05, **P<0.01, significantly different from each control value obtained in cells with Control siRNA).

4.3.2. *Sox6* and *Col II* transcriptional activity was upgraded

In contrast, under the same experimental conditions, a significant and sustained increase in *Sox6* mRNA expression was observed, along with the increased mRNA levels of *Col II* (Figure 4.2), in ATDC5 cells cultured for 36 to 60 h after transfection.

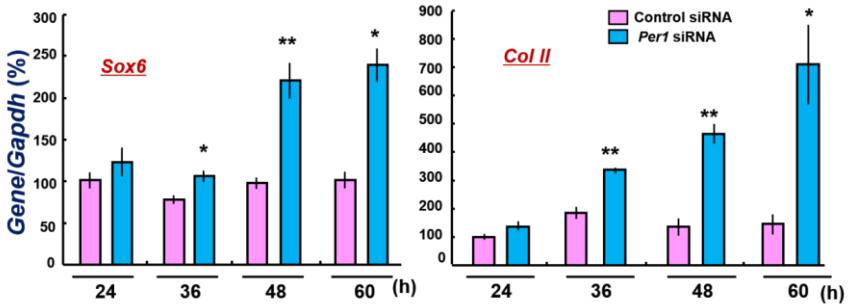

Figure 4.2 Effect of *Per1* knockdown on ATDC5 cells. (*P<0.05, **P<0.01, significantly different from each control value obtained in cells with Control siRNA).

4.4. Conclusion

The outcome of experiment on *Per1* knock down partially by suppressing its mediators, mRNA, to translational protein turned out to be as expected and was in favor of *Per1* suppression role on both *Sox6* and detection of *Col II* expression reduction nearly in a temporal correlation.

CHAPTER 5. CLOCK GENE EXPRESSION AFFECTS CHONDROGENESIS

5.1. Analysis of protein – DNA interaction in E-box binding motif

CLOCK/BMAL1 heterodimer mediates transcriptional activation of target genes or clock-controlled genes (CCGs) via cognate E-box sequence (a consensus sequence of CACGTG) lying within these genes' promoters as a set of *cis*-regulatory enhancer tandems. The E-Box plays an important role in transactivation of circadian genes (Bozek et al., 2007).

5.2. Methods

5.2.1. *Sox6* promoter design

Mouse *Sox6* promoter was at first obtained by cloning with the forward primer 5'-GGTACC (KpnI site)-ATGGGCTGGCTTTGAAAACT-3' (-2,190 to -2,171) and the reverse primer 5'-CTCGAG (XhoI site)-CAGAGAATTAATCTAAACA -3' (+181 to +200) with mouse tail genomic DNA. The deletion mutant of mouse *sox6* promoter plasmid was made from the forward primer 5'-GGTACC (KpnI site)-AATTGTTGTTACTTTCTA-3' (-1,080 to -1,061) and the reverse primer 5'-CTCGAG (XhoI site)-CAGAGAATTAATCTAAACA -3' (+181 to +200). (Fig 5.1).

5.2.2. Luciferase assay: performed as described in 3.4.3.

5.2.3. PCR

PCR was performed using sequences found in the 5'-flanking region of *sox6* promoter fragment, which were cloned into the promoterless pGL-3 basic vector.

5.2.4. ATDC5 culture: performed as described in **2.2.1**.

5.2.5. Chromatin Immunoprecipation (ChIP) assay

Immunoprecipitation was performed with the anti-BMAL1 (Rabbit, Santa Cruz) antibody followed by extraction of DNA with phenol/chloroform.

5.3. Results

5.3.1. Effects of different clock combine to *Sox6* promoter activity

In HEK293 cells with *Sox6*-Luc, the introduction of *Bmal1* and/or *Clock* led to a significant increase in luciferase activity in a manner sensitive to the inhibition by *Per1* transfection. However, luciferase activity was not significantly affected by the introduction of *Bmal1*/*Clock* in cells with *Sox6*-Luc (Fig.5.1).

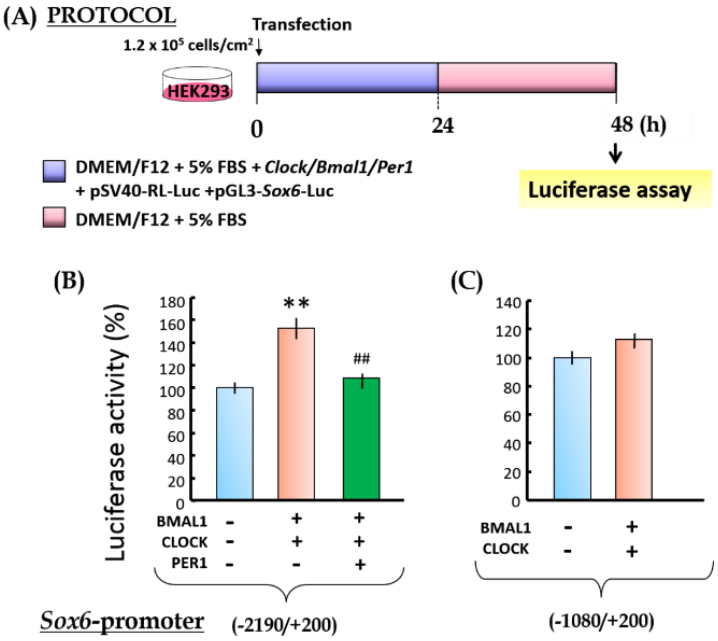

Figure 5.1. Cotransfection of *Clock/Bmal1/Per1* into HEK293 cells following **(A)** protocol with **(B)** *Sox6*-full length promoter-Luc and **(C)** *Sox6*-deleted promoter-Luc. (*P<0.05, **P<0.01, significantly different from each control value obtained in cells with EV. ($^{\#\#}$P<0.01, significantly different from the value obtained in cells with *Bmal1/Clock*).

5.3.2. Effect of *Per1* upregulation on *Sox6* promoter activity

Luciferase activity was significantly decreased in ATDC5-*Per1* #28 cells transfected with full *Sox6*-Luc compared to ATDC5-EV cells 48 h after transfection. Transient overexpression of *Per1* led to a similarly significant reduction of luciferase activity in ATDC5 cells with *Sox6*-Luc (Fig.5.2).

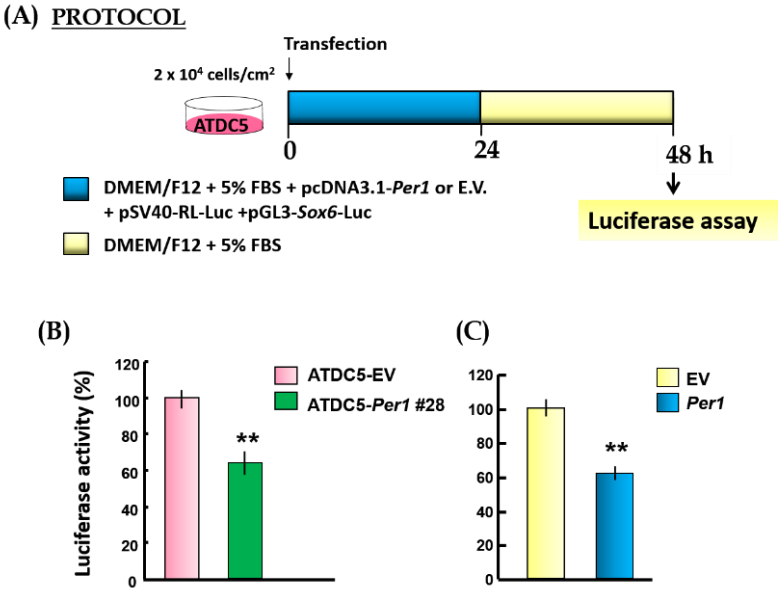

Figure 5.2. *Sox6* promoter activity: **(A)** Protocol; **(B)** Stable transfectant cells with *Sox6* promoter-Luc, and **(C)** ATDC5 cells were transiently transfected with *Sox6* promoter-Luc. (*P<0.05, **P<0.01, significant differences compared with EV).

5.3.3. Clock gene *Per1* expression and *Sox6* transactivation

PCR product from lysate of ATDC5 cells transfected with *Bmal1*/*Clock* was clearly seen within region containing an E-box element (-1388/-1184) but not with that devoid of the E-box element (+242/+540) of the *Sox6* gene (Fig.5.3).

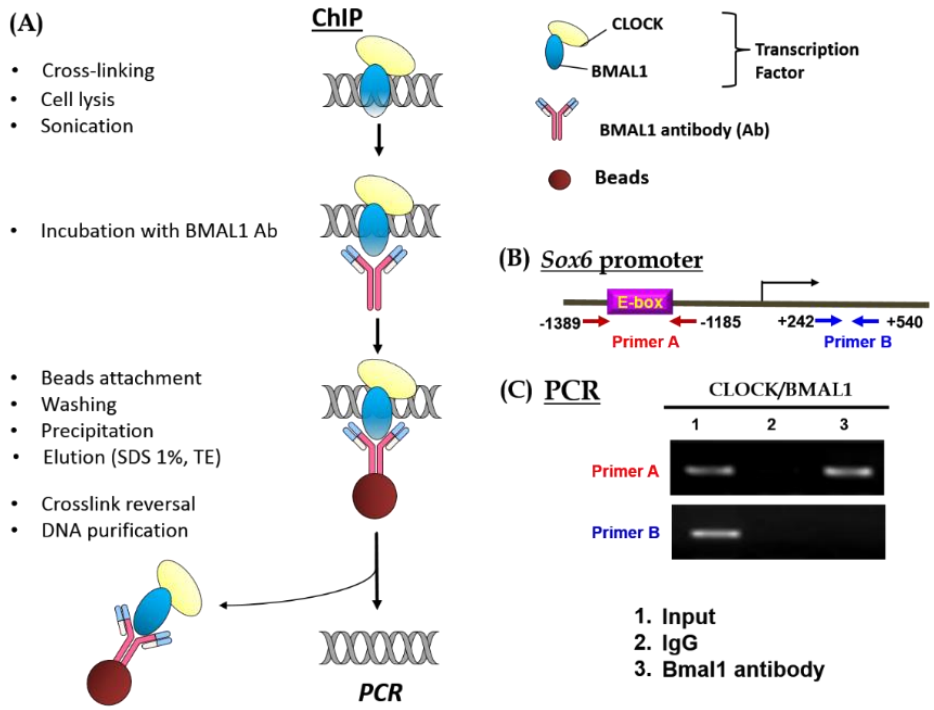

Figure 5.3. ATDC5 cells were transfected with *Bmal1, Clock* and *Per1* expression vectors, followed by **(A)** immunoprecipitation with the anti-Bmal1 antibody and subsequent **(B, C)** RT-PCR using either primers A or primers B.

5.4. Conclusions

Per1 expression disrupted chondrogenesis by inhibiting CLOCK/BMAL1 transactivation of *Sox6*.

CHAPTER 6. DISCUSSION

6.1. Summary

- Successfully established a gain/loss-in-function model of clock gene *Per1* in pre-chondrogenic ATDC5 cells;
- Directly demonstrated that *Per1* mediated *Sox6* downregulation by inhibiting CLOCK/BMAL1 dimer activity of the core circadian loop.

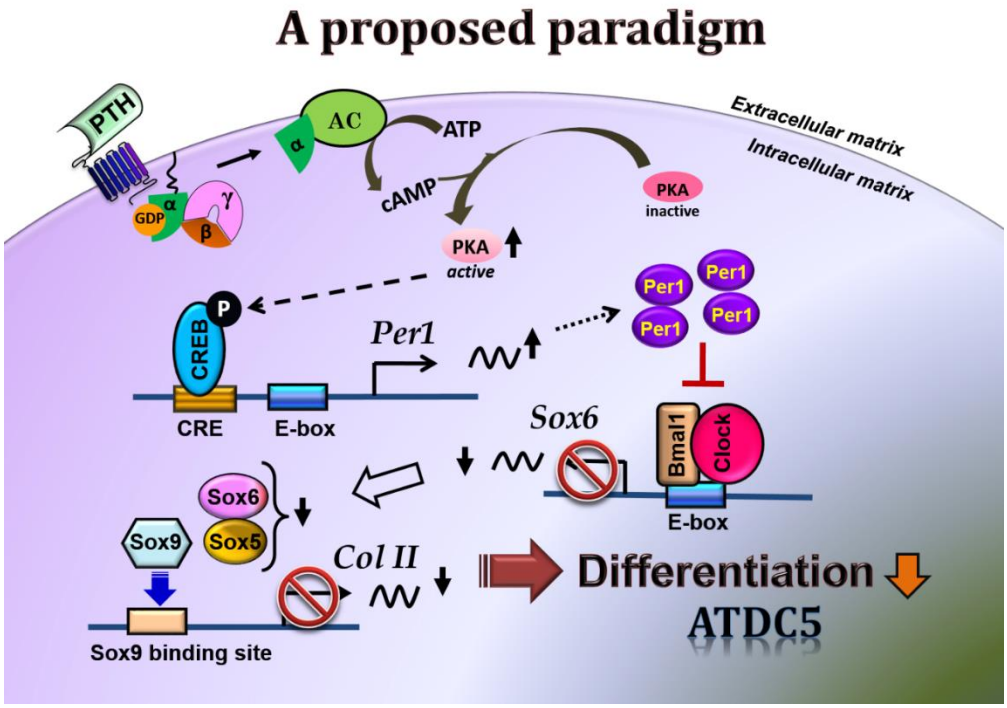

Figure 6.1. A proposed model of *Per1* interference to chondrogenesis.

6.2. Critical remarks

To date, this is the first direct demonstration of a negative correlation between *Per1* and *Sox6* expression at the level of gene transactivation in pre-chondrocytic ATDC5 cells. *Sox6* activity partial failure also reduced that of Sox-trio including *Sox9*. Besides, deficiency of *Sox9* gene expression results in a severe skeletal malformation syndrome namely campomelic dysplasia (Bi et al., 2001) while a transgenic mice overexpressing of *Sox9* showed the delayed endochondral bone formation (Takarada et al., 2008). Thus, clock gene *Per1* possibly mediated a retardation in chondrocyte differentiation. Nevertheless, the final conclusion should await the demonstration of failure of *Per1* to suppress chondrogenic differentiation in chondrocytes isolated from mice defective of *Per1*.

With the concept of the circadian clock as a nature's tool to effectively deal with the daily routine, our finding could help putting therapeutic interventions against cartilaginous cartilage diseases up to a wrinkle by figuring out how time-varying influences cartilaginous growth in human.

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学位論文審査報告書（甲）

1．学位論文題目（外国語の場合は和訳を付けること。）

TIME PACING IN CHONDROGENESIS MEDIATED BY THE CLOCK GENE PERIOD 1

（時計遺伝子ピリオド１による軟骨細胞分化の周期的制御）

2．論文提出者　(1) 所　　属　生命科学　専攻　分子作用学　講座

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3．審査結果の要旨（600～650 字）

本研究では、時計遺伝子の一つであるピリオド１(Per1)について、軟骨細胞における機能的発現の可能性を in vitro 細胞培養実験系を用いて追究した。軟骨分化を抑制する副甲状腺ホルモン(PTH)を、前軟骨細胞株である ATDC5 細胞に 48 時間持続的に曝露すると、軟骨分化制御因子の一つである *Sox6* 遺伝子発現の持続的低下とともに、*Per1* 遺伝子発現の一過性上昇が観察されたが、他の軟骨分化制御因子 *Runx2* 遺伝子の発現には著明な変化は見られなかった。新規樹立の *Per1* 安定発現 ATDC5 細胞では、*Sox6* 遺伝子発現の劇的低下だけでなく、分化指標である II 型コラーゲン(*Col II*)遺伝子発現が抑制されたが、*siRNA* により *Per1* 遺伝子発現を低下させると、*Sox6* と *Col II* の両遺伝子発現が逆に著明に上昇した。HEK293 細胞に *Sox6* 遺伝子プロモーター連結のルシフェラーゼレポーターを導入すると、*Bmal1/Clock* 両遺伝子導入により有意な活性上昇と Per1 導入に伴う阻害が観察された。*Per1* 安定発現 ATDC5 細胞では、*Sox6* プロモーター活性が強く阻害されたが、ChIP アッセイの結果 *Sox6* プロモーター上流-1389 bp の E-box 配列に Bmal1 蛋白が結合する事実が確認された。したがって、PTH による軟骨細胞の分化成熟抑制には、時計遺伝子 *Per1* 発現上昇を介する *Sox6* 遺伝子の転写抑制が少なくとも一部は関与すると推察される。以上の研究成績は、副甲状腺ホルモン PTH の軟骨分化抑制作用の出現メカニズムに、軟骨細胞に発現する時計遺伝子 *Per1* が直接的に関与する可能性を初めて提唱しただけでなく、変形性関節症や慢性関節リウマチ等の関節疾患の予防と治療を指向する創薬戦略への貢献が期待される点で高く評価されるので、審査委員会は本論文が博士（薬学）に値すると判断した。

4．審査結果　(1) 判　　定（いずれかに○印）　(合　格)　・　不合格

(2) 授与学位　博　士（　薬学　）